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OBSERVATIONS ON THE RATE OF GROWTH OF B. COLI

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This work was planned originally to determine quantitatively the effect of anilin dyes and of different mediums employed for the isolation of B. coli on the rate of growth of this organism.

The studies undertaken include:

1. The normal rate of growth of B. coli in peptone water at 37 C.
2. The effect of the age of the culture on the rate of growth.
3. The effect of crystal violet and brilliant green.
4. The rate of growth in various standard mediums.
5. The effect of varying amounts of bile salts.
6. The effect of varying amounts of peptone.
7. The effect of dipotassium phosphate.
8. The effect of Liebig's beef extract.

HISTORICAL

The work of several investigators has shown that the rate of growth of an organism varies during the life of the culture when kept under constant conditions of temperature, light, and other factors of environment. These variations in the rate of growth divide the life of the culture into distinct periods.

Buchanan[1] divides the life cycle of a culture into 7 phases, each phase having a different rate of growth per organism than the phase next preceding it.

The phases are as follows:

1. The initial stationary phase. During this phase the number of bacteria remains constant or nearly so.
2. The lag phase or positive growth acceleration phase. During this phase the average rate of growth per organism increases with the time.
3. The logarithmic growth phase. The rate of growth per organism in this period is constant. The organisms are dividing regularly and the number of organisms in the culture is increasing in a geometric ratio.
4. The phase of negative growth acceleration. The average rate of growth per organism decreases during this period.

Received for publication Oct. 28, 1918.

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[1] Jour. Infect. Dis., 1918, 23, p. 109.

5. The maximum stationary phase. There is little or no increase or decrease in the number of organisms during this period.
6. The phase of accelerated death. The number of bacteria decreases slowly at first, but the rate of death per organism gradually increases until it reaches a maximum.
7. The logarithmic death phase. During this phase the rate of death per organism is constant.

From these observations, it is apparent that any investigation of the rate of growth of organisms must take into account these various growth phases. A given factor may influence the rate of growth in one phase and not in another. It may cause a lengthening of the lag phase and have no influence on the logarithmic growth phase, or may even stimulate growth during the latter phase.

Barber[2] has shown that the age of the culture influences the lag phase. He found that if a subculture is made in the same kind of medium during the logarithmic period, this subculture does not go through a lag period, but continues to grow at the same rate per organism as the parent culture. If the subculture is made after the logarithmic period, however, there is distinct lag. Penfold[3] and later Chesney[4] confirmed these observations. The lag phase, that is, the time from the inoculation to the beginning of the logarithmic phase, according to these investigators, includes the first two phases as outlined by Buchanan.

Rahn[5] found that an increase in the numbers caused a shortening of the lag phases, when comparatively small numbers were used for inoculation.

Chick[6] concludes from experiments with B. coli that the previous history of the culture greatly influences the rate of growth during the initial period. She found that harmful effects were produced by prolonged incubation of the culture at 37 C., or by exposure to borax, or to fresh milk. Injury to the cells in various ways caused a period of lag when the organisms were transferred to a favorable medium.

Lane-Claypon[7] determined the generation time for B. coli and for B. enteriditis (Gaertner) at various temperatures. He found that generation time is shortened by increases in temperature up to 42 C., and that these variations in rate of growth follow the Arrhenius-Van't Hoff law. His results in regard to generation time of B. coli were in accord with those of Barber.[2]

METHOD

The method of measuring the effect of various factors on the rate of growth of organisms was suggested by the frequently reported observation of investigators that the rate of growth per organism during the logarithmic phase is constant. If the logarithms of the numbers of viable organisms during intervals of this period are plotted against time, the logarithms fall along an ascending straight line. A given strain of organism should give similar curves if the conditions of growth are exactly reproduced in each case. With this fact well established, the effect of various factors might be noted by the change which they produce in the slope of the line. We would expect a stimulating substance to increase the slope and an inhibiting substance to decrease the slope of the line. In other words, the tangent of the angle which the curve makes with the X axis, compared with the normal, could be taken as a measure of the influence of the substance or factor.

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[2] Jour. Infect. Dis., 1908, 5, p. 379.
[3] Jour. Hyg., 1914, 14, p. 215.
[4] Jour. Exper. Med., 1916, 24, p. 387.
[5] Centralbl. f. Bakteriol., 1900, 16, p. 417.
[6] Jour. Hyg., 1912, 12, p. 414.
[7] Jour. Hyg., 1909, 9, p. 239.

Perhaps a more convenient method of measuring and expressing the effect of the factors on the rate of growth is that used by Chesney.[4] He expressed the rate of growth in terms of average generation time. The generation time is the time required by an organism to grow to maturity and divide. In other words, it is the time required to produce a single generation. This can be computed by a formula worked out by Buchner, Longard, and Riedlin.[8]

It is assumed that during the logarithmic period the organisms are increasing in a geometrical ratio. By the law of geometrical proportions: $b = B\ 2^n$, where B is the number of bacteria at the beginning and B is the number of bacteria after time t, and n is the number of generations. If there have been n generations in time t, then $g = \frac{t}{n}$, where g is generation time.

$$\text{Then } n = \frac{t}{g}$$

$$\text{and } b = B\ 2\frac{t}{g}$$

Applying logarithms to this equation:

$$\text{Log } b = \log B + \log 2\frac{t}{g}$$

$$g = \frac{t \log 2}{\log b - \log B}$$

When time t is not too long the generation time can be estimated with considerable accuracy, but since the rate of growth per organism changes at the beginning and at the end of the logarithmic phase, the number of organisms must be determined at frequent intervals.

The method employed was to grow the organisms at a constant temperature in a simple liquid medium, determine the numbers present at frequent intervals of time, and compute the generation time. The effect of variations in the medium and other factors could then be determined by the change which they produce in the generation time. The advantage of this method is that it shows on which phase of the life of the culture the effect is most marked.

The medium chosen for this work was a one-half per cent. Difco peptone-distilled water solution. All of the peptone for these experiments was taken from the same bottle to insure uniformity in composition. It was sterilized in test tubes in the autoclave at 15 pounds pressure for 15 minutes. This medium was stored in an icebox, but never kept more than 3 or 4 days on account of change in concentration due to evaporation.

The organism used was a well tested strain of B. (coli) communis. This was grown in the peptone water and transferred daily for 3 successive days before each experiment. This was done to avoid any changes in the rate of growth due to previous environment of the culture. Each experiment was begun with a 22-24 hour culture, except where otherwise stated.

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[8] Centralbl. f. Bakteriol., 1887, 11, p. 1.

The temperature of incubation during these experiments was kept constant by means of a DeKhotinsky electrically controlled water-bath. By the use of this apparatus the temperature could easily be kept constant within 1 degree.

The determinations of the number of organisms present at various periods of growth were made by plating on plain agar. This medium was prepared in large quantities and great care taken to insure uniformity of composition. Four plates were made for each determination. Dilutions, when necessary, were made in sterile tap water. The exposure to the water was never for more than a few minutes and apparently it had no effect on the subsequent development of the organism.

The plates were counted after 48 hours of incubation at 37° C. In making the counts a hand lens was used which magnified about 5 diameters. All plates which could not be counted accurately were discarded.

THE RATE OF GROWTH OF B. COLI IN PEPTONE WATER

An experiment was planned to determine the rate of growth per organism of B. coli in peptone water and to determine roughly the limits of the logarithmic period as a working basis for following experiments. Four tubes, each containing 10 c c of peptone water, were inoculated: 2 with B. communis and 2 with B. communior. One-tenth c c of a 1: 10,000 dilution of a 24-hour culture was added in each tube. This gave between 200 and 300 organisms per c c. These were incubated in the water-bath and counts made at the beginning and at the end of each 3-hour period. The results are shown in Table 1.

TABLE 1

RATE OF GROWTH OF B. COLI IN PEPTONE WATER

	Time in Minutes	Average Number Bacteria per C C	Generation Time in Minutes
B. communis	0	175	
	180	4,550	38.5
	360	639,000	25.3
	540	57,100,000	27.8
B. communis	0	158	
	180	5,600	35.0
	360	585,000	26.8
	540	48,000,000	28.4
B. communior*	0	83	
	180	1,350	44.8
	360	379,000	22.1
	540	44,500,000	26.2

* The plates of the second culture of B. communior showed contamination and, therefore, the results are not included in the table.

It will be noted that in each case the first 3 hours show considerably slower growth than the following periods, due to a lag phase. The fact that the last 3 hours always show a lengthening of the generation time would indicate that this time included a part, at least, of the phase of negative growth acceleration.

The mean generation time for B. coli at 37 C., as determined by Barber[2] was 17-21 minutes. Lane-Claypon[7] reports similar results. Both these investigators used plain broth as a culture medium.

In order to get a better view of the various phases of growth under the conditions of this experiment, it was repeated using B. communis and making a count every 2 hours. This experiment was continued for 12 hours. The results are shown in Table 2.

TABLE 2

RATE OF GROWTH OF B. COLI IN PEPTONE WATER

Time in Minutes	Average Number Bacteria per C C	Generation Time in Minutes
0	233	
120	600	88.0
240	79,450	17.2
360	1,360,000	29.3
480	22,200,000	29.8
600	448,000,000	27.8
720	1,489,000,000	69.4

The 2-hour period shows the slow rate of growth at the beginning more distinctly since it more nearly represents the initial and lag phases. The very short generation time between the 2nd and 4th hours is probably an experimental error. Repeated counts in subsequent experiments failed to show such a short period of rapid growth as this figure would indicate. The rate of growth in peptone water at 37 C. is apparently constant from the 4th to the 10th hour after inoculation. After the 10th hour the lengthening of the generation time is quite marked.

THE EFFECT OF AGE OF CULTURE

As previously stated, Barber[2] noted that subcultures from cultures which are still in the logarithmic phase, show no initial stationary or lag phase. An experiment was made to check this observation. Subcultures were made from rapidly growing cultures after 4 hours of incubation and cultures and subcultures were counted at the end of 4 additional hours. The results shown in Table 3 would indicate the rate of growth of the subcultures closely approximates that of the parent culture.

It is apparent that the age of the culture influences the rate of growth within certain limits. In order to determine the best age of culture for the experiments which follow, the generation times were determined for 6 cultures varying in age from 1-6 days. The cultures had been kept in the incubator at 37 C. The results of this experiment (Table 4) are quite striking. The variations in the generation time with cultures of varying ages appear to be almost entirely within the first 2 hours. A glance at the generation times in this period will show that the stationary and lag phases are not greatly increased until the 3rd day is passed and then the increase in these phases is very decided.

TABLE 3

THE RATE OF GROWTH OF SUBCULTURES MADE DURING THE LOGARITHMIC PHASE

Time in Hours	Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	B. communis	263	
4		10,900	42.5
8		2,980,000	31.5
4	Subculture	1,000	
8		144,000	36.1
0	B. communis	305	
4		9,450	51.5
8		2,570,000	31.5
4	Subculture	945	
8		238,500	31.9
0	B. communior	230	
4		56,200	32.2
8		18,195,000	30.6
4	Subculture	5,620	
8		2,625,000	28.8
0	B. communior	231	
4		19,500	39.8
8		5,022,000	33.2
4	Subculture	1,950	
8		890,000	28.9

If we accept the view of Coplans[9] that the slow rate of initial growth is due to previous injury to the cells caused by products of metabolism, it would seem that some decided change has taken place in the culture between the 3rd and 4th days.

Buchanan's explanation of the lag is that bacterial cells, like seeds of higher plants, go into a resting stage and when placed again in favorable environment, they require a certain length of time in which to germinate. Interpreting the results from this viewpoint, we must

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[9] Jour. Pathol., 1909, 14, p. 1.

TABLE 4

THE EFFECT OF AGE OF CULTURE ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Age of Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	One Day	388	
2		1,070	72.1
4		13,000	33.3
6		177,000	31.8
8		4,400,000	25.9
0	Two Days	538	
2		1,520	80.1
4		19,500	32.6
6		550,000	24.9
8		9,800,000	28.9
0	Three Days	338	
2		930	82.2
4		29,700	24.0
6		680,000	26.6
8		6,100,000	37.9
0	Four Days	349	
2		520	179.7
4		7,500	26.9
6		167,000	23.1
8		1,750,000	30.5
0	Five Days	271	
2		461	134.9
4		5,300	29.4
6		77,500	26.7
8		800,000	30.7
0	Six Days	262	
2		330	310.6
4		6,800	23.7
6		93,000	27.5
8		1,420,000	26.2

SUMMARY OF GENERATION TIMES

Time in Hours	One Day	Two Days	Three Days	Four Days	Five Days	Six Days
0–2	72.1	80.1	82.2	179.7	134.9	310.6
2–4	33.3	32.6	24.0	26.9	29.4	23.7
4–6	31.8	24.9	26.6	23.1	26.7	27.5
6–8	25.9	28.9	37.9	30.5	30.7	26.2

conclude that a large number of cells go into the resting stage after the 3rd day. This explanation is quite as reasonable as that based on Coplans' theory.

In order to get at the changes which take place in cultures during the initial phases, the generation times of 1- and of 6-day cultures were determined at 30 minutes' intervals, with the results shown in Table 5. The 6-day culture shows both a lengthening of the lag period and an increase in the generation time during this period over the 1-day culture. The 1-day culture has nearly reached its maximum rate of growth in 1 hour. Graphs 1 and 2 illustrate the difference in the rates of growth of the 2 organisms. Graph 1 shows the constancy of the rate of growth of cultures after the lag period and shows also that the rate of growth during the logarithmic period is independent of the age of the culture.

TABLE 5

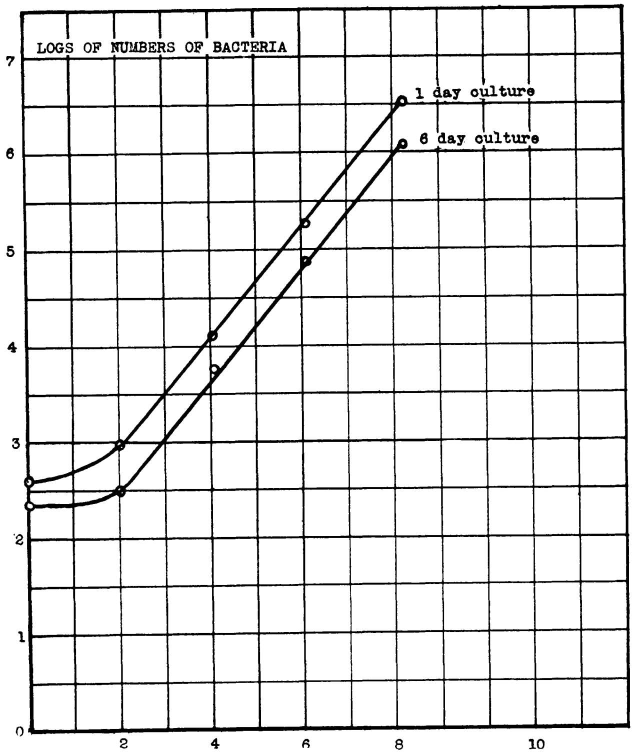
THE EFFECT OF AGE OF CULTURE ON RATE OF GROWTH OF B. COMMUNIS

Time in Minutes	Age of Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	One Day	30	
30		37	99.1
60		55	52.4
90		105	32.2
0	Six Days	37	
30		41	202.4
60		52	87.5
90		70	69.9

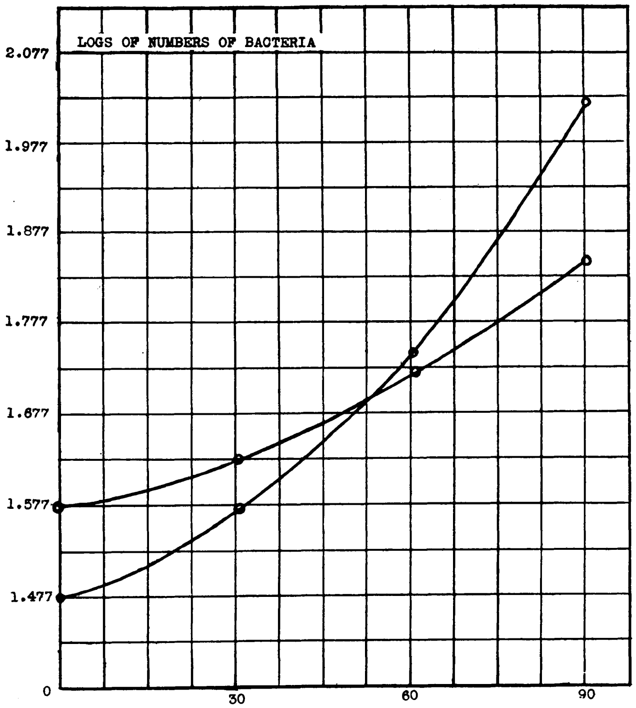
SUMMARY OF GENERATION TIMES

Time in Minutes	One Day	Six Days
0–30	99.1	202.4
30–60	52.4	87.5
60–90	32.2	69.9

Graph 2 shows that in the 1-day culture there is growth from the beginning, and if there is a stationary phase at all, it must be extremely short. The 6-day culture also shows growth from the beginning, but the rate of acceleration of growth is decidedly slower than in the 1-day culture. From the results of this experiment it seemed that a 24-hour culture would be best to use for succeeding experiments.



Graph 1.—Showing the rate of growth of a one- and six-day culture.



Graph 2.—Effect of age of culture on lag.

THE EFFECT OF CRYSTAL VIOLET AND BRILLIANT GREEN

It has been known for some time that anilin dyes effect the growth of bacteria. Billroth was the first to note this effect, although Stilling[10] was the first to use anilin dyes in a practical way as antiseptics. Churchman[11] and others have noted that certain dyes have a selective action on bacteria.

Gentian violet, according to Churchman,[11] can be used to divide bacteria roughly into 2 groups, one of which will grow readily in the presence of this dye, and the other will be entirely inhibited by it. The first group corresponds to the gram-negative bacteria and the second to the gram-positive bacteria. Charles Krumweide and Josephine Pratt[12] confirmed this work of Churchman's on the selective action of dyes. They found that occasionally a gram-negative organism refused to grow on violet agar, often differentiating it from members of the same species or variety. They found a marked difference in the growth of dysentery and paradysentery, especially on fuchsin agar. Diphtheria and diphtheroid bacteria acted differently in the presence of naphthalamin blue.

All of this work was based on the growth of the organism on solid mediums containing the dye, and the effect of the dye was read by the relative abundance of growth on the dye agar compared with the plate agar controls.

Kriegler[13] attempted to find some correlation between the chemical structure of the dyes and their disinfecting properties. He also noted a marked variation in the resistance of closely related organisms.

Nowhere in the literature was found any attempt at an accurate quantitative measure of the effect of dyes on growth. If great variations in the lethal dilutions for related organisms exist, and if distinc tions can be readily made between growth of related organisms on agar cultures containing the dyes, it would seem that an accurate quantitative measure of the inhibiting effect of weak dilutions of dyes might be of value.

CRYSTAL VIOLET

A stock 10% solution of the dye in distilled water was prepared and from this a series of dilutions in peptone water was made. The stock solution was tightly stoppered and kept in the ice-box to prevent evaporation. The dilutions in peptone water were prepared fresh for each experiment.

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[10] Berlin. klin. Wchnschr., 1890, 24; Arch. f. Exper. Path. u. Pharmakol., 1891, 28, p. 357.

[11] Jour. Exper. Med., 1913, 17, p. 373.

[12] Jour. Exper. Med., 1914, 19, p. 20 and p. 501.

[13] Centralbl. f. Bakteriol., I, O., 1911, 59, p. 481.

Tubes containing 10 c c of the various dilutions of dyes in peptone water, and one control tube containing peptone water only, were placed in the water bath for 15 minutes previous to making inoculations in order to bring the mediums to proper temperature. At the end of 4 hours' growth, a count was made on all of the tubes and a second series of the same dilutions of dyes was inoculated from the control tube. This was to determine whether the effect of the dye would be the same on a rapidly growing culture as on the fresh transfers. At the end of 8 hours, the number of bacteria per c c in all tubes was determined. Several preliminary experiments failed to give good results until the proper dilution of dyes and the proper dilutions for the plate counts were established. It was found that growth took place in dilutions of 1:200,000. In strong dilutions growth was very irregular or absent.

It was noted that growth appeared in a particular dilution on one day while on another day, it was absent or very slight. It would appear that the action of the dyes varied on different days when all controllable environmental factors seemed to be constant. The action of the dyes seemed to be strongest on bright sunshiny days. It was suggested that the variable factor might be the light.

Table 6 shows a comparison of generation times on different days. The fact that the rate of growth of the controls is constant on different days within the limit of experimental error, would point to the fact that the dyes themselves were more active on one day than on another. It was suggested that the action of varying amounts of light on a fluorescent dye might influence its growth inhibiting power.

TABLE 6

GENERATION TIME ON DIFFERENT DAYS

Dilution of Dye	Period of Growth in Hours	Nov. 6	Nov. 8	Nov. 14	Nov. 22	Nov. 28	Dec. 8	Dec. 13
Control, no dye	0–4	35.2	35.7	35.4	37.0	....	34.8	32.8
	4–8	30.1	27.3	26.8	25.1	....	27.3	28.9
1:75,000	0–4	decrease	569.0					
	4–8	decrease	decrease					
1:100,000	0–4	decrease	477.0					
	4–8	30.2	131.3					
1:200,000	0–4	90.6	.....	96.8	32.6	.....	62.0	124.1
	4–8	47.4	.....	.....	92.9	.....	41.6	44.2
1:400,000	0–4	58.7	55.8	53.3	49.6	.....	44.2	55.6
	4–8	27.8	77.1	36.9	61.5	.....	55.0	33.0
1:600,000	0–4	.....	.....	40.5	51.1	59.3	39.8	38.4
	4–8	.....	.....	31.4	35.1	29.4	29.6	33.4
1:800,000	0–4	.....	.....	34.2	36.9	555.3	39.8	38.4
	4–8	.....	.....	28.8	41.1	37.5	29.6	333.3
1:1,000,000	0–4	.....	.....	.....	.....	56.0	37.4	36.5
	4–8	.....	.....	.....	.....	28.0	28.2	32.3

Von Tappeiner,[14] Raah,[15] and others have shown that organisms in water solutions of fluorescent dyes behave differently in the presence of light than they do in the dark. Raab investigated the effect of a

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[14] Deutsch. Arch. f. klin. Med., 1904, 80, p. 1904.

[15] Inaug. Diss. München, 1900.

TABLE 7

THE EFFECT OF CRYSTAL VIOLET ON THE RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	393	
4	Control	46,300	34.8
8	Control	17,400,000	27.2
4	1:200,000	5,740	62.0
8	1:200,000	304,000	41.6
4	1:400,000	17,000	44.2
8	1:400,000	348,000	55.0
4	1:600,000	208,000	41.7
8	1:600,000	4,880,000	30.5
4	1:800,000	25,600	39.8
8	1:800,000	7,080,000	29.6
4	1:1,000,000	31,400	37.4
8	1:1,000,000	11,400,000	28.2
4	Subculture 1:200,000	34,800	67.0
4	Subculture 1:400,000	82,800	57.6
4	Subculture 1:600,000	81,000	58.0
4	Subculture 1:800,000	86,000	52.7
4	Subculture 1:1,000,000	127,000	50.2

SUMMARY OF GENERATION TIMES

Time	1:200,000	1:400,000	1:600,000	1:800,000	1:1,000,000	Control
0-4	62.0	44.2	41.7	39.8	37.4	34.8
4-8	41.6	55.0	30.5	29.6	28.2	27.2
Subculture	67.0	57.6	58.0	52.7	50.0	

TABLE 8

THE EFFECT OF CRYSTAL VIOLET ON THE RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	390	
4	Control	62,500	32.8
8	Control	19,750,000	28.9
4	1:200,000	1,490	124,1
8	1:200,000	64,300	44.2
4	1:400,000	10,410	50.6
8	1:400,000	1,710,000	33.0
4	1:600,000	20,300	42.7
8	1:600,000	3,120,000	33.3
4	1:800,000	29,800	38.4
8	1:800,000	4,400,000	33.3
4	1:1,000,000	37,000	36.5
8	1:1,000,000	6,350,000	32.3
4	Subculture 1:200,000	34,900	96.7
4	Subculture 1:400,000	111,000	57.8
4	Subculture 1:600,000	174,000	50.0
4	Subculture 1:800,000	200,000	48.0
4	Subculture 1:1,000,000	274,000	44.0

SUMMARY OF GENERATION TIMES

Time	1:200,000	1:400,000	1:600,000	1:800,000	1:1,000,000	Control
0-4	124.1	50.6	42.7	38.4	36.5	32.8
4-8	44.2	33.0	33.3	33.3	32.3	28.9
Subculture	96.7	57.8	50.0	48.0	44.0	

number of fluorescent substances on various organisms. He found that many organisms were killed when exposed to daylight in fluorescent solutions, while in the dark they lived indefinitely. Von Tappeiner found that fluorescent dyes will also destroy the activity of toxins and antitoxins when exposed to the light. After a somewhat extended study of fluorescent substances, Von Tappeiner concluded that the photodynamic effect is associated with those rays which are absorbed by the fluorescent materials. He also concluded that in order to bring about the photodynamic action, the material in question must be visibly fluorescent even when examined in the rays of a mercury vapor lamp.

It would seem then that an explanation of the variations in the rate of growth on different days based on the photodynamic action of the dyes when exposed to lights of different intensity, would be inadequate without further experimental evidence.

Tables 6 and 7 show the results of two experiments with crystal violet which are quite comparable. It will be noted that the dye has its greatest inhibiting power during the first 4 hours. After that the figures for the generation times are more nearly like those of the control. Evidence that the dyes are active in producing a lag phase is shown by the fact that the subcultures from the controls into the dyes do not grow at nearly the same rate as the cultures which have been exposed to the dyes for a 4-hour period. In most cases the lag produced in the subcultures is greater than the lag produced in the 24-hour cultures from which the first inoculations were made. It would seem that the actively growing cells were more susceptible to injury than the cells in an older culture.

BRILLIANT GREEN

Two experiments were made with brilliant green, identical with those performed with crystal violet, except that higher dilutions of the dye were used. Preliminary experiments showed this dye to be much more inhibitive than crystal violet. The results are shown in Tables 9 and 10. In general, they are the same as those for stronger solutions of crystal violet. Brilliant green does not seem to affect the lag period more than it does the logarithmic period, but it does produce a marked lag in subcultures from an actively growing culture, as did the crystal violet.

TABLE 9

The Effect of Brilliant Green on Rate of Growth of B. communis

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	274	
4	Control	72,000	28.0
8	Control	27,340,000	22.0
4	1:600,000	70	
8	1:600,000	55	
4	1:800,000	240	
8	1:800,000	245	826.0
4	1:1,000,000	550	299.0
8	1:1,000,000	1,750	144.0
4	1:2,000,000	2,940	129.0
8	1:2,000,000	1,700	
4	Subculture 1:600,000	1,160	
4	Subculture 1:800,000	26,700	91.0
4	Subculture 1:1,000,000	12,000	325.0
4	Subculture 1:2,000,000	178,000	55.8

SUMMARY OF GENERATION TIMES

Time	1:600,000	1:800,000	1:1,000,000	1:2,000,000	Control
0–4	No growth	No growth	239	129	28
4–8	No growth	826	144	. . .	22
Subculture	No growth	91	325	55.8	

TABLE 10

The Effect of Brilliant Green on Rate of Growth of B. communis

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	213	
4	Control	70,000	28.7
8	Control	29,000,000	27.6
4	1:2,000,000	5,630	50.8
8	1:2,000,000	24,000	113.1
4	1:4,000,000	6,600	45.4
8	1:4,000,000	81,000	66.3
4	1:6,000,000	14,400	39.4
8	1:6,000,000	1,390,000	36.3
4	1:10,000,000	61,600	29.3
8	1:10,000,000	34,900,000	26.2
4	Subuclture 1:2,000,000	37,900	99.9
4	Subculture 1:4,000,000	47,000	87.3
4	Subculture 1:6,000,000	121,000	58.3
4	Subculture 1:10,000,000	322,000	43.4

SUMMARY OF GENERATION TIMES

Time in Hours	1:2,000,000	1:4,000,000	1:6,000,000	1:10,000,000	Control
0–4	50.8	45.4	39.4	29.3	28.7
4–8	113.1	66.3	36.3	26.2	27.6
Subculture	99.9	87.3	58.3	43.4	

Both crystal violet and brilliant green, because of their inhibiting influence on the members of the colon group and other organisms, are used in the isolation of members of the typhoid group. Further investigation of these dyes concerning their effect on other members of the colon group should be made. The present work will be confined to B. (coli) communis and a study of the other factors on its rate of growth.

TABLE 11

The Effect of Various Culture Mediums on Rate of Growth of B. communis

Time in Hours	Medium	Average Number Bacteria per C C	Generation Time in Minutes
0	½% Peptone	282	
2		1,490	49.9
4		44,100	24.5
8		20,800,000	27.0
2	Peptone K_2HPO_4	4,200	30.8
4		192,000	21.7
8		38,100,000	31.4
2	Peptone bile salt	......	....
4		10,600	45.9*
8		1,510,000	33.4
2	Standard broth	2,210	43.9
4		76,000	23.5
8		40,500,000	41.8
2	Lactose broth	1,300	54.6
4		37,800	22.1
8		33,700,000	26.0

SUMMARY OF GENERATION TIMES

Time in Hours	½% Peptone	Peptone K_2HPO_4	Peptone Bile Salt	Standard Broth	Lactose Broth
0-2	49.9	30.8	....	43.9	54.6
2-4	34.5	21.7	45.9*	23.5	22.1
4-8	27.0	31.4	33.4	41.8	26.0

* Determined for 4 hour period.

THE EFFECT OF VARIOUS STANDARD MEDIUMS

A large number of liquid mediums are used for the cultivation of B. coli, for preliminary enrichment, isolation, presumptive tests, and tests for the presence of metabolic products. The rates of growth in a few of these mediums were determined in order to detect the presence of inhibiting or stimulating substances and to get a comparison of the rates of growth.

The following mediums were used:

1. Peptone water: One-half per cent. peptone in distilled water.
2. Peptone-dipotassium phosphate: One-half per cent. peptone water plus two-tenths per cent. dipotassium phosphate.
3. Peptone bile salt: One-half per cent. peptone to 1% of commercial bile salt.
4. Broth: Prepared according to "Standard Methods of Water Analysis," except that the reaction was made neutral to phenolphthalein before sterilization.
5. Lactose broth: Prepared according to "Standard Methods of Water Analysis."

TABLE 12

THE EFFECT OF VARIOUS MEDIUMS ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Medium	Average Number Bacteria per C C	Generation Time in Minutes
0	Standard peptone	225	
2		875	61.5
4		27,200	24.3
8		22,800,000	25.5
2	Peptone K_2HPO_4	1,790	40.3
4		95,500	21.0
8		52,200,000	27.2
2	Peptone bile salt	700	73.8
4		13,800	28.0
8		9,250,000	26.4
2	Standard broth	960	57.4
4		46,900	21.4
8		37,100,000	25.7
2	Lactose broth	875	61.5
4		38,500	22.1
8		26,400,000	26.3

SUMMARY OF GENERATION TIMES

Time in Hours	½% Peptone	Peptone K_2HPO_4	Peptone Bile Salt	Standard Broth	Lactose Broth
0-2	61.5	40.3	73.8	57.4	61.5
2-4	24.3	21.0	28.0	21.4	22.1
4-8	25.5	27.2	26.4	25.7	26.3

The hydrogen-ion concentrations of all these mediums were determined by colorimetric comparison with standards, and they were all found to have a P_H value of between 6.5 and 7.6. This amount of variation is probably within the favorable range of concentration for the growth of B. coli, or at least these concentrations would not be likely to cause any inhibitions of growth. Clark has some unpublished work on the effect of hydrogen-ion concentration. In a personal letter to Prof. Max Levine, Jan. 24, 1918, he states that "the most striking results which we obtained were, first: the broad P_H zone within which the maximum rate of reproduction during the period of logarithmic increases was practically constant."

Tables 11 and 12 contain the results of duplicate experiments to show the rate of growth of B. coli in various mediums. A glance at the maximum numbers of organisms reached in the various mediums will show that the standard broth and the peptone phosphate mediums yield the most abundant growth, while the lactose broth, peptone water, and the bile salt mediums follow in the order named. It is apparent that beef extract and dipotassium phosphate greatly stimulate the growth of B. communis. In the case of the dipotassium phosphate this stimulation appears to be quite marked in the lag period, while in the standard broth and lactose broth, whose stimulating ingredient is undoubtedly the beef extract, the greatest stimulation appears during the second 2 hours of growth. This would indicate that the stimulating effects of dipotassium phosphate and beef extract were not identical, and that the stimulation of beef extract was not entirely due to its phosphate content.

A very decided inhibition of growth is caused by the addition of bile salt. Not only does it produce a lengthened lag period, but it increases the generation time throughout the period of growth. Since bile salts are used in the presumptive test for B. coli, a further study of the effect of this substance was made.

THE EFFECT OF BILE SALTS

Prescott and Winslow[16] state: "By far the most satisfactory results in making a rapid test for the colon group have been obtained by the use of mediums containing bile salts." Dunschmann[17] studied a favorable action of bile on B. typhosus and a slight inhibition on B. coli. Echer[18] states: "Bile salts distinctly retard the growth of typhoid bacilli." Most of the work seems to have been done with whole bile rather than with bile salts. Hirokawa[19] reports that B. coli, B. typhosus, and B. Paratyphosus A and B develop well in bile mediums. Frankel and Krause[20] report that human bile also stimulates the growth of typhoid bacilli, B. coli, Staphylococcus pyogenes, and Vibrio cholerae. On the other hand, Pies[21] found a sample of human bile which inhibited both B. coli and B. typhosus. Nichols[22] states that rabbit bile in vitro may be antiseptic to B. typhosus, B. paratyphoid A, B. coli, and B. dysenteriae. Jordan[23] asserts: "Bile inhibits at least from one-third to one-half of the viable cells of B. coli and sometimes a much larger proportion." Obst[24] similarly states that lactose bouillon permits the development of about twice as many coli as will lactose peptone ox-bile medium.

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[16] Principles of Water Bacteriology, 1913.
[17] Ann. de l'Inst. Pasteur, 1909, 23, p. 29.
[18] Jour. Exper. Med., 1918, 22, p. 95.
[19] Centralbl. f. Bakteriol., I, O., 1909, 53, p. 12.
[20] Ztschr. f. Hyg., 1899, 32, p. 91.
[21] Inaug. Diss. Strassburg, 1907.
[22] Jour. Exper. Med., 1916, 24, p. 497.
[23] Jour. Infect. Dis., 1913, 12, p. 326.
[24] Jour. Bacteriol., 1916, 1, p. 73.

From the literature, it is apparent that there is lack of agreement regarding the effect of bile on B. coli and related organisms.

The present study was confined to different quantities of bile salts. Dilutions of commercial bile salt in peptone water were prepared and the generation times for B. coli for the various dilutions were determined. It is evident from the results of these experiments that small quantities of bile salts, that is, under five-tenths per cent., stimulate the growth of B. communis, while greater amounts, such as 1%, greatly inhibit growth. This may, in a measure, explain the disagreement in the results of other investigators. The bile and bile salts from various sources undoubtedly contained varying amounts of the stimulating substances with the result that some found stimulation and others found inhibition. Several investigators have reported a selective action on the part of bile and bile salts, and there seems to be need of investigating the effect of varying amounts of bile salt on other members of the colon group.

TABLE 13

THE EFFECT OF DIFFERENT CONCENTRATIONS OF BILE SALT ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Bile Salt	Average Number Bacteria per C C	Generation Time in Minutes
0	All	124	
2	Control	410	70.0
4	Control	12,800	24.3
8	Control	14,600,000	23.7
2	0.1	595	58.7
4	0.1	17,900	24.6
8	0.1	23,700,000	23.2
2	0.2	515	58.7
4	0.2	17,900	23.5
8	0.2	36,600,000	21.9
2	0.3	450	64.8
4	0.3	17,000	23.0
8	0.3	24,700,000	22.8
2	0.5	520	58.2
4	0.5	20,200	22.8
8	0.5	21,500,000	23.9
2	0.7	425	67.8
4	0.7	13,400	24.2
8	0.7	10,800,000	24.9
2	1.0	435	66.7
4	1.0	7,150	29.9
8	1.0	1,650,000	30.5

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	0.7	1.0
0–2	70.0	53.2	58.7	64.8	58.2	67.8	66.7
2–4	24.3	24.6	23.5	23.0	22.8	24.2	29.9
4–8	23.7	23.2	21.9	22.8	23.9	24.9	30.5

THE EFFECT OF DIPOTASSIUM PHOSPHATE

As was noted in the foregoing, the addition of two-tenths per cent. of dipotassium phosphate to peptone water greatly stimulated growth. Clark and Lubs[25] recommend the use of a peptone dipotassium phosphate medium for testing for various reactions of B. coli. Levine[26] used dipotassium phosphate instead of beef extract in a modified Endo medium which he found very successful. The dipotassium phosphate acts as a hydrogen-ion buffer and obviates the necessity of careful adjustment of the reaction.

TABLE 14

THE EFFECT OF DIFFERENT CONCENTRATIONS OF BILE SALT ON THE GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Bile Salt	Average Number Bacteria per C C	Generation Time in Minutes
0	All	250	
2	Control	1,250	49.3
4	Control	31,200	25.9
8	Control	32,600,000	24.0
2	0.1	1,330	47.7
4	0.1	46,800	23.4
8	0.1	33,800,000	25.4
2	0.2	1,480	44.3
4	0.2	50,600	23.6
8	0.2	......	....
2	0.3	1,600	43.1
4	0.3	56,300	23.6
8	0.3	36,000,000	25.8
2	0.5	1,600	43.1
4	0.5	58,300	23.2
8	0.5	15,100,000	30.1
2	0.7	1,130	52.5
4	0.7	38,600	23.6
8	0.7	6,760,000	32.2
2	1.0	1,180	51.0
4	1.0	23,000	28.1
8	1.0	1,780,000	38.4

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	0.7	1.0
0-2	49.3	47.7	44.3	43.1	43.1	52.5	51.0
2-4	25.9	23.4	23.6	23.6	23.2	23.6	28.1
4-8	24.0	25.4	....	25.8	30.1	32.2	38.4

An experiment was planned to determine what amount of dipotassium phosphate would give the most rapid rate of growth of B. communis. Various amounts of phosphate were carefully weighed and dissolved in portions of the same batch of peptone water. These solutions were then tubed and sterilized. The generation times were determined in the usual manner.

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[25] Jour. Bacteriol., 1917, 2, p. 1.

[26] Jour. Am. Pub. Health Assn., 1918, 8, p. 866.

The results shown in Tables 15 and 16 would indicate that the rate of growth of B. communis is stimulated by dipotassium phosphate in amounts up to 1% in one-half per cent. peptone water.

TABLE 15

THE EFFECT OF DIPOTASSIUM PHOSPHATE (K_2HPO_4) ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. K_2HPO_4	Average Number Bacteria per C C	Generation Time in Minutes
0	All	223	
2	Control	1,150	52.2
4	Control	26,400	26.5
6	Control	770,000	24.7
8	Control	18,300,000	26.3
2	0.1	1,300	48.4
4	0.1	24,800	28.2
6	0.1	620,000	25.9
8	0.1	19,600,000	24.1
2	0.2	880	62.5
4	0.2	23,400	25.4
6	0.2	865,000	23.1
8	0.2	21,800,000	25.7
2	0.3	1,020	56.5
4	0.3	30,500	24.5
6	0.3	650,000	27.2
8	0.3	26,200,000	22.5
2	0.5	840	64.9
4	0.5	16,000	26.2
6	0.5	650,000	22.4
8	0.5	29,000,000	21.9
2	1.0	1,770	41.0
4	1.0	72,300	22.4
6	1.0	3,220,000	21.9
8	1.0	39,400,000	33.2

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	1.0
0–2	52.2	48.4	62.5	56.5	64.9	41.0
2–4	26.5	28.2	25.4	24.5	26.2	22.4
4–6	24.7	25.9	23.1	27.2	22.4	21.9
6–8	26.3	25.2	25.7	22.5	21.9	33.2

The two tables are not in exact agreement; but of the two, the first gives the more consistent results and therefore is probably more nearly correct. A rise in the generation time may be seen in the 6-8 hour period with the higher concentration of phosphate in both experiments. This is, undoubtedly, an indication that the end of the logarithmic growth period has been reached before the 8th hour and thus the rate shows a decline. The lag period is markedly decreased by the greater concentrations of phosphate. On the whole, the effect of dipotassium phosphate seems to be to cause a production of the maximum number of organisms in a shorter time.

THE EFFECT OF VARIOUS AMOUNTS OF PEPTONE

The Committee on Standard Methods of the American Public Health Association, in their 1917 report have reduced the amount of peptone in standard mediums for water analysis from 1% to one-half per cent. An experiment was made to determine the effect of different amounts of peptone on the rate of growth of B. communis. The mediums were prepared by weighing out different amounts of peptone and dissolving it in distilled water, tubing it, and sterilizing it at 15 lbs. pressure for 15 minutes. The generation times of B. communis in the different concentrations of peptone were determined in the usual manner. Tables 17 and 18 show the results.

TABLE 16

THE EFFECT OF DIPOTASSIUM PHOSPHATE (K_2HPO_4) ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. K_2HPO_4	Average Number Bacteria per C C	Generation Time in Minutes
0	All	462	
2	Control	1,090	97.0
4	Control	27,050	25.9
6	Control	558,000	27.4
8	Control	16,650,000	24.4
2	0.1	1,570	68.0
4	0.1	38,700	26.0
6	0.1	1,380,000	23.2
8	0.1	37,300,000	25.2
2	0.2	1,180	88.5
4	0.2	27,500	26.4
6	0.2	1,290,000	21.6
8	0.2	19,400,000	30.7
2	0.3	1,560	68.5
4	0.3	24,700	30.1
6	0.3	111,000	21.8
8	0.3	20,500,000	15.9
2	0.5	2,340	51.3
4	0.5	59,500	25.7
6	0.5	3,420,000	20.5
8	0.5	36,500,000	35.1
2	1.0	2,280	52.2
4	1.0	75,000	23.8
6	1.0	3,570,000	21.5
8	1.0	16,300,000	54.8

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	1.0
0-2	97.0	68.0	88.5	68.5	51.3	52.2
2-4	25.9	26.0	26.4	30.1	25.7	23.8
4-6	27.4	23.2	21.6	21.8	20.5	21.5
6-8	24.4	25.2	30.7	15.9	35.1	54.8

It is evident that the amount of peptone has a marked influence on the rate of growth. The total number of organisms increases with the concentration of peptone up to 5%. The two experiments are not in agreement on the 5% concentration, but the figure in the first is based on a count of over 500 colonies per plate and is probably not accurate. The figure in the second experiment, based on a very satisfactory count, is undoubtedly more nearly the true number.

TABLE 17

THE EFFECT OF CONCENTRATION OF PEPTONE ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Peptone	Average Number Bacteria per C C	Generation Time in Minutes
0	0.5	175	
2	0.5	730	58.2
4	0.5	8,760	33.4
6	0.5	170,000	28.0
8	0.5	2,940,000	29.2
2	1.0	840	53.0
4	1.0	10,800	32.6
6	1.0	204,000	28.3
8	1.0	3,280,000	29.9
2	2.0	1,090	45.5
4	2.0	335,100	23.9
6	2.0	940,000	25.3
8	2.0	31,500,000	23.7
2	3.0	1,300	41.4
4	3.0	79,000	20.1
6	3.0	1,750,000	26.9
8	3.0	60,900,000	23.2
2	4.0	1,500	38.3
4	4.0	148,000	18.1
6	4.0	2,740,000	28.6
8	4.0	134,200,000	21.2
2	5.0	1,800	35.3
4	5.0	123,000	19.7
6	5.0	2,430,000	27.9
8	5.0	99,900,000	22.2

SUMMARY OF GENERATION TIMES

Time in Hours	0.5	1.0	2.0	3.0	4.0	5.0
0–2	58.2	53.0	45.5	41.4	38.3	35.3
2–4	33.4	32.6	23.9	20.1	18.1	19.7
4–6	28.0	28.3	25.3	26.9	28.6	27.9
6–8	29.2	29.9	23.7	23.2	21.2	22.2

It will be seen that the 5% peptone water gives the most rapid growth. A 2% peptone, however, gives a great increase in numbers over a 1% peptone, and where a very rapid growth is desired, this amount of peptone might be profitably used.

It is quite remarkable that the increased rate of growth with the greater concentrations of peptone is largely in the lag period, or, in other words, the organisms reach their maximum rate of growth more quickly in the concentrated peptone water.

TABLE 18

The Effect of Concentration of Peptone on Rate of Growth of B. communis

Time in Hours	Per Cent. Peptone	Average Number Bacteria per C C	Generation Time in Minutes
0	0.5	257	
2	0.5	630	94.2
4	0.5	18,200	24.3
6	0.5	263,000	31.1
8	0.5	4,390,000	29.5
2	1.0	970	62.6
4	1.0	27,400	24.8
6	1.0	705,000	25.3
8	1.0	11,400,000	29.8
2	2.0	1,580	45.8
4	2.0	49,100	24.2
6	2.0	1,710,000	23.4
8	2.0	66,000,000	22.8
2	3.0	1,830	42.4
4	3.0	63,100	23.5
6	3.0	3,140,000	21.3
8	3.0	127,000,000	22.3
2	4.0	1,660	44.5
4	4.0	98,200	20.4
6	4.0	5,000,000	21.2
8	4.0	194,000,000	22.7
2	5.0	1,360	50.0
4	5.0	80,000	19.3
6	5.0	5,300,000	19.8
8	5.0	270,000,000	20.4

SUMMARY OF GENERATION TIMES

Time in Hours	0.5	1.0	2.0	3.0	4.0	5.0
0–2	94.2	62.6	45.8	42.4	44.5	50.0
2–4	24.3	24.8	24.2	23.5	20.4	19.3
4–6	31.1	25.3	23.4	21.5	21.2	19.8
6–8	29.5	29.8	22.8	22.3	22.7	20.4

THE EFFECT OF BEEF EXTRACT

It was thought best to try out the effect of the three-tenths per cent. beef extract recommended by the Committee on Standard Methods of the American Public Health Association, and compare the rate of growth in it with that in mediums containing five-tenths per cent. beef extract, and with another containing no beef extract. These amounts of beef extract were added to one-half per cent. peptone water, and the generation times were determined as in preceding experiments. The results (Table 19) show that three-tenths per cent. beef extract gives a much greater growth than no beef extract. A 48-hour culture was used in this experiment, which accounts for the excessive lag.

Beef extract decreases both the lag period and the generation time during the logarithmic period. Five-tenths per cent. of beef extract gives a shorter lag period than three-tenths per cent., but on the whole the difference between the rate of growth in the five-tenths per cent. and the three-tenths per cent. is not very great, as is shown by the total number of organisms.

TABLE 19

THE EFFECT OF BEEF EXTRACT ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Beef Extract	Average Number Bacteria per C C	Generation Time in Minutes
0	All	358	
2	Control	510	235.0
4	Control	8,000	30.1
6	Control	111,000	31.6
8	Control	1,770,000	30.6
2	0.3	480	284.0
4	0.3	9,200	28.2
6	0.3	319,000	23.4
8	0.3	12,700,000	22.6
2	0.5	525	217.0
4	0.5	13,500	25.6
6	0.5	320,000	26.2
8	0.5	14,450,000	21.8

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.3	0.5
0-2	235.0	284.0	217.0
2-4	30.1	28.2	25.6
4-6	31.6	23.4	26.2
6-8	30.6	22.6	21.8

CONCLUSIONS

After a period of lag, the rate of growth of B. communis at 37 C. in one-half per cent. Difco peptone is constant and at a maximum from 6-8 hours.

Subcultures, during the period of constant rate of growth, as previously shown by Barber and others, do not show lag.

Crystal violet decreases the rate of growth of B. communis in dilutions as great as 1 : 1,000,000. The effect of the dye is greatest during the lag period.

B. communis will not grow in one-half per cent. Difco peptone water containing a concentration of crystal violet greater than 1:200,000. A slight inhibition of growth is caused by a dilution of 1:1,000,000.

Brilliant green in dilutions of 1:600,000 inhibits the growth of B. communis in one-half per cent. Difco peptone. Inhibition disappears between dilutions of 1:6,000,000 and 1:10,000,000. A slight stimulation is noted at 1:10,000,000.

Crystal violet and brilliant green have a greater inhibitive effect on rapidly growing cultures than on old cultures. The action of dyes varies on different days.

Bile salts stimulate the growth of B. communis when added to one-half per cent. peptone in dilutions as high as three-tenths to five-tenths per cent. Higher concentrations of bile salts inhibit growth. One per cent. shows marked inhibition.

Increasing the concentration of peptone up to 5% stimulates the rate of growth of B. communis. The lag period is shortened in the greater concentrations of peptone.

Addition of dipotassium phosphate to peptone in amounts up to 1% accelerates the rate of growth of B. communis.

Addition of beef extract to peptone increases the rate of growth of B. communis; five-tenths per cent. giving slightly more rapid growth than three-tenths per cent.

The age of the culture greatly influences the rate of growth of B. communis during the lag period. Both the generation times and the duration of the lag are greater in old cultures. A marked increase in lag appears after 3 days.